A Novel Multipeptide Microarray for the Specific and Sensitive Mapping of Linear IgE-Binding Epitopes of Food Allergens

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Abstract: BACKGROUND The identification of B-cell epitopes of food allergens can possibly lead to novel diagnostic tools and therapeutic reagents for food allergy. We sought to develop a flexible, low-tech, cost-effective and reproducible multipeptide microarray for the research environment to enable large-scale screening of IgE epitopes of food allergens. METHODS Overlapping peptides (15-mer, 4 amino acid offset) covering the primary sequence of either peanut allergen Ara h 1 or all 3 subunits of the soybean allergen Gly m 5 were simultaneously synthesized in-house on a porous cellulose matrix. Identical peptide microarrays created with up to 384 duplicate peptide-cellulose microspots each were investigated for specificity and sensitivity in IgE immunodetection and in direct experimental comparison to the formerly established SPOT™ membrane technique. RESULTS The in-house microarray identified with 98% reproducibility the same IgE-binding peptides as the SPOT™ membrane technique. Additional IgE-binding peptides were identified using the microarray. While the sensitivity was increased between 2- and 20-fold, the amount of human serum required was reduced by at least two thirds over the SPOT™ membrane technique using the microarray. After subtraction of the potential background, we did not observe non-specific binding to the presented peptides on microarray. CONCLUSIONS The novel peptide microarray allows simple and cost-effective screening for potential epitopes of large allergenic legume seed storage proteins, and it could be adapted for other food allergens as well, to study allergenic epitopes at the individual subject level in large paediatric and adult study groups of food allergic subjects.

DOI: https://doi.org/10.1159/000381344

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2015;166:213–224
DOI: 10.1159/000381344

Received: December 22, 2014
Accepted after revision: February 27, 2015
Published online: April 24, 2015

A Novel Multipeptide Microarray for the Specific and Sensitive Mapping of Linear IgE-Binding Epitopes of Food Allergens

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Key Words
Epitope mapping · Peptides · Microarray · Food allergens · Immunoglobulin E · Soybean allergen · Peanut allergen · SPOT™ membrane

Abstract
Background: The identification of B-cell epitopes of food allergens can possibly lead to novel diagnostic tools and therapeutic reagents for food allergy. We sought to develop a flexible, low-tech, cost-effective and reproducible multipeptide microarray for the research environment to enable large-scale screening of IgE epitopes of food allergens. ***Methods:*** Overlapping peptides (15-mer, 4 amino acid offset) covering the primary sequence of either peanut allergen Ara h 1 or all 3 subunits of the soybean allergen Gly m 5 were simultaneously synthesized in-house on a porous cellulose matrix. Identical peptide microarrays created with up to 384 duplicate peptide-cellulose microspots each were investigated for specificity and sensitivity in IgE immunodetection and in direct experimental comparison to the formerly established SPOT™ membrane technique. ***Results:*** The in-house microarray identified with 98% reproducibility the same IgE-binding peptides as the SPOT™ membrane technique. Additional IgE-binding peptides were identified using the microarray. While the sensitivity was increased between 2- and 20-fold, the amount of human serum required was reduced by at least two thirds over the SPOT™ membrane technique using the microarray. After subtraction of the potential background, we did not observe non-specific binding to the presented peptides on microarray. ***Conclusions:*** The novel peptide microarray allows simple and cost-effective screening for potential epitopes of large allergenic legume seed storage proteins, and it could be adapted for other food allergens as well, to study allergenic epitopes at the individual subject level in large paediatric and adult study groups of food allergic subjects.

Introduction

Food allergy, an IgE antibody-mediated and potentially life-threatening [1] chronic immunological disease, is a significant clinical problem in Europe and appears to have increased within the past years [2]. Based on clinical food challenge data, peanut and soybean are among the most prevalent elicitors of allergic reactions to foods in Europe [3].

Epitope mapping of B-cell epitopes, i.e. the immunoglobulin-binding structures of proteins, is a fundamental tool to understand the antigen-antibody interaction of the

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human immune response. B-cell epitopes can be classified into two categories: (i) discontinuous epitopes where two or more binding regions are discontinuously located in the primary structure and full antibody reactivity depends on the intact conformation of the antigen and (ii) continuous or linear epitopes, merely consisting of the conformation of the primary amino acid sequence [4]. Considering the harsh conditions of food technological processing and protein digestion in the human gastrointestinal tract, complete native structural integrity may play a minor role in immunoglobulin binding to cause clinical reactivity in food allergy. Based on observations that short stretches of classical food allergens retain at least partial IgE-binding capacity, several studies aimed at identifying IgE-binding stretches that are involved in the allergic disease by screening against an array of synthetic overlapping peptides that cover the complete primary allergen sequence. Accordingly, diagnostic markers for severe allergic reactions [5] or persistent food allergy may be identified [6, 7]. In addition, knowledge of the B-cell epitopes of allergens allows designing hypoallergenic immunotherapeutic peptides of allergenic molecules [8, 9] or hypoallergenic variants of allergens for immunotherapy [10].

To date, synthetic peptide arrays for food allergen analysis have largely been generated on cellulose membranes with SPOT™ peptide synthesis, which gives the opportunity to synthesize a large number of individual peptides in parallel [11–13]. Detailed information about the most common IgE-binding epitopes of a food allergen can only be obtained by screening a large number of sera from allergic patients at the individual subject level. Unfortunately, the SPOT™ membrane technique is not particularly suitable as a screening tool for such analyses, mainly because the synthesis and immunodetection of a large number of peptides is time consuming and requires high quantities of chemicals and especially precious and limited patients' sera. Hence, various different peptide microarrays have been developed to reduce the amount of serum required. As additional key features for food allergen epitope analysis resolved at the individual subject level, such a peptide microarray would need to allow the simultaneous screening of identical array replicates of even large proteins that consist of several subunits, such as the peanut and soybean major storage proteins. A common design of peptide microarrays for allergen analysis has been the separate synthesis of overlapping peptides following automated spotting or printing onto modified surfaces of arrays slides [14–20].

In the field of food allergen IgE epitope analysis, one peptide microarray appeared to be well validated [20]. It was based on commercially synthesized overlapping peptides which were printed onto epoxy-derivatized glass slides. The binding of human serum IgE was recorded by fluorescence detection using an array reader. In our study, we aimed at developing a multipeptide microarray having comparable performance characteristics while, for reasons of cost-efficiency and flexibility, setting up an in-house multipeptide synthesis and a simple and cost-effective microarray spotting and detection procedure. We chose the Celluspot™ array platform that makes use of peptides covalently synthesized on a cellulose porous membrane which builds a 3-dimensional structure that is spotted and attached onto a standard microscope slide [21]. The method has already been applied for determining antibody specificities to phosphotyrosin [22] or histone tail posttranslational modifications [23]. In this study, we investigated the potential of the multipeptide microarray in comparison to the well-established SPOT™ membrane technology [11, 13] for its handling, sensitivity and specificity in the identification of human IgE-binding epitopes of legume seed storage proteins related to the allergic disease. Allergen peptide-specific human IgE was detected by chemiluminescence on X-ray film, and signals were analysed with open source array software after digitalization. As an example, overlapping 15-mer peptides of either the peanut major allergen Ara h 1 [24] or all 3 subunits of the homologous allergenic soybean major storage protein β-conglycinin [25], namely Gly m 5.01 (α), Gly m 5.02 (α′) and Gly m 5.03 (β), were analysed using both the SPOT™ membrane and our in-house microarray method. The results demonstrate that this novel, simple and cost-effective peptide microarray is suitable for the identification of allergenic epitopes at the individual subject level in large paediatric and adult study groups of food allergic subjects.

Methods

Human Sera

Inclusion criteria for human subjects were a clinically confirmed food allergy to soybean on the basis of a convincing history of anaphylaxis to soybean (No. 28 and 8) [26] or a positive oral challenge with soybean (No. 25). All subjects included had a convincing history of peanut allergy. Specific IgE (sIgE) to soy and peanut extract and to recombinant peanut allergen Ara h 1 as well as to natural soybean allergen Gly m 5 was quantified using the ImmunoCAP™ system (Thermo Fisher Scientific, Uppsala, Sweden). As a negative control, a serum pool consisting of equal volumes of sera from 6 non-allergic (NA) human subjects from an in-house serum collection was applied. Sera with sIgE levels >0.35 kU_A/l are considered positive. Further details are given in table 1.

Table 1. Characteristics of human serum samples from peanut- and soybean-allergic patients and NA control

Patient No.	Food allergy	Specific IgE according to ImmunoCAP™ analysis, kU_A/l			
		Ara h 1	peanut	Gly m 5	soybean
28	Peanut, soybean	>100	>100	23.10	52.5[1]
8	Peanut, soybean	<0.35	2.30[1]	n.d.	15.9[1]
25	Peanut, soybean	>100	>100	13.30	30.0
NA	NA	n.d.	n.d.	n.d.	n.d.

NA denotes the sera pool with 6 NA subjects from the Paul-Ehrlich-Institut (PEI), Division of Allergology, in-house serum collection (PEI-19, -208, -230, -231, -233, -245). n.d. = Not determined.

[1] Ballmer-Weber et al. [26], 2007.

Table 2. Investigated allergenic 7S seed storage proteins from peanut and soybean: protein identity and number of array peptides (15 amino acids length, 4 amino acid offset)

Allergen	Protein	UniProt entry	Sequence coverage (aa)	Array peptides, n
Ara h 1	Peanut vicilin	P43237	26–614	145
Gly m 5.01	Soybean β-conglycinin, α subunit	O22120	1–543	133
Gly m 5.02	Soybean β-conglycinin, α' subunit	Q9FZP9	1–559	137
Gly m 5.03	Soybean β-conglycinin, β subunit	P25974	24–439	102

Allergens are defined according to the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (www.allergen.org). The investigated amino acid sequence covers the mature protein primary sequence without signal peptide (according to Maruyama et al. [42], 1998). aa = Amino acid.

Synthesis of Overlapping Peptides on Cellulose SPOT™ Membranes

Arrays of overlapping peptides (15 amino acids, offset of 4 amino acids) of the primary sequence of the mature forms of peanut allergen Ara h 1 and the 3 subunits of the homologous soybean allergen Gly m 5, namely Gly m 5.01 (α subunit), 5.02 (α' subunit) and 5.03 (β subunit), were synthesized according to the principles of Merrifield [27]. Using the SPOT™ technique [11, 13], the simultaneously synthesized peptides were covalently bound to an amino-PEG_{500} derivative cellulose membrane (Intavis AG, Cologne, Germany) using an automated multiple peptide synthesizer with up to 600 peptide spots per membrane according to the manufacturer's instructions (MultiPep, Intavis AG). After peptide synthesis, the side chain protection groups of the amino acids were deprotected with a mixture of dichloromethane/trifluoroacetic acid/triisopropylsilane/water in a ratio of 1:1:0.06:0.04. The N terminus was blocked by acetylation. According to the manufacturer of the multiple peptide synthesizer, the purity of the peptides should be better than 70%. Membranes with synthesized peptides were stored under dry conditions at 4°C until use. Details about the proteins and the derived synthetic overlapping peptides are given in table 2. Physical details about the SPOT™ membranes are listed in table 3. Figure 1 displays 2 SPOT™ membranes that present 145 Ara h 1-related peptides which were immunostained by the NA serum pool and the serum No. 28 of a peanut- and soybean-allergic donor, respectively.

Synthesis and Preparation of Overlapping Celluspot™ Peptides for Microarrays

Arrays of overlapping synthetic peptides (15 amino acids, offset of 4 amino acids) of Ara h 1, Gly m 5.01, 5.02 and 5.03 were synthesized as described above except for an Fmoc-β-alanine etherified cellulose membrane (Intavis AG) as the solid phase. After peptide synthesis, all peptide spots were cut from the membrane using a puncher (Intavis). Each membrane disc carrying an individual peptide was dissolved over night with 250 µl of a mixture of trifluoroacetic acid/trifluoromethanesulphonic acid/triisopropylsilane/water (89.5:4:2.5:4). Thereafter, 750 µl of –20°C cold tert-butylmethylether were added to each peptide, and the peptides were stored at –70°C for 1 h. The precipitated peptides were centrifuged at 2,000 *g* for 10 min at 0°C. The ether supernatant was removed, and the peptide pellet was washed twice each with 500 µl of –20°C cold tert-butylmethylether and centrifuged at 2,000 *g* for 10 min at 0°C. After ether removal, the peptide pellet was dissolved in 300 µl dimethylsulphoxide (DMSO). An aliquot of 50 µl of each dissolved peptide was pipetted into dedicated wells of a 384-well ELISA plate.

Generation of Peptide Microarrays

Using a slide-spotting robot (Intavis AG), the DMSO stocks of the overlapping 15-mer peptides of peanut Ara h 1 and the 3 subunits of soybean Gly m 5 were spotted onto microscope slides (26 × 76 mm) coated with white adhesive foil (Intavis AG) in 2 identical

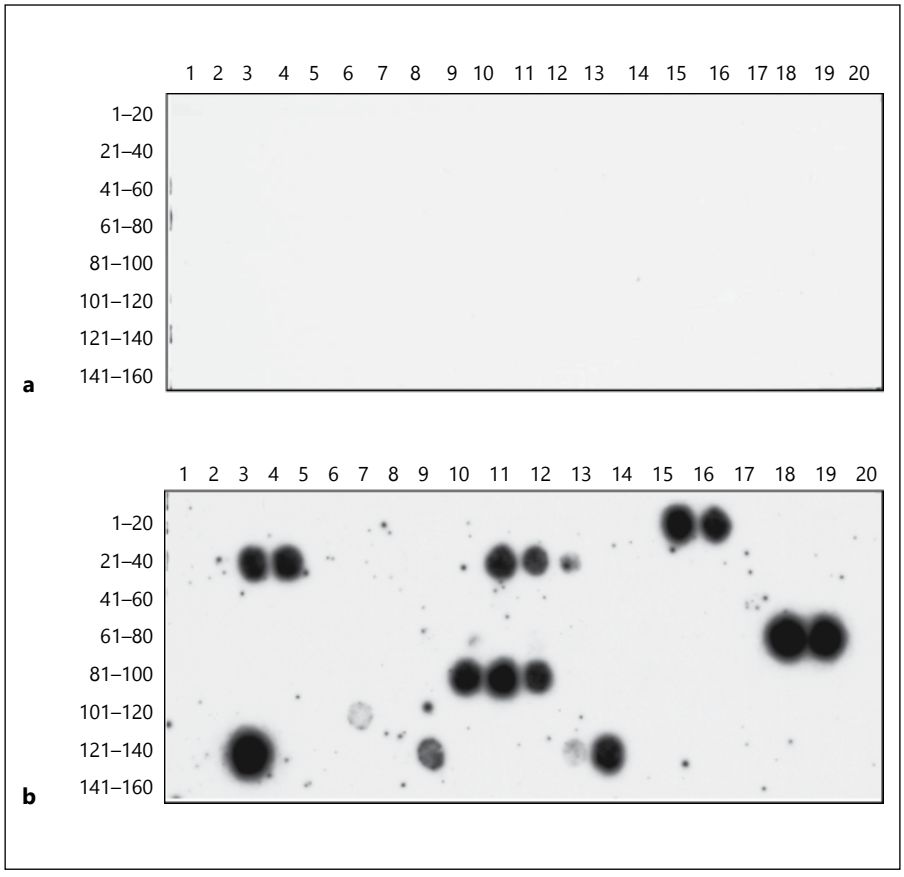


Fig. 1. SPOT™ membranes for the IgE epitope mapping of peanut allergen Ara h 1. IgE immunodetection of 145 overlapping synthetic peptides using a serum pool of 6 NA subjects (**a**) and serum of peanut- and soybean-allergic subject No. 28 (**b**). Consecutive numbers indicate the position of individual peptides.

Table 3. Characteristics of SPOT™ membrane and Celluspot™-based microarrays in this study

Parameter	SPOT™ membrane (A)	Microarray (B)	Ratio B/A
Max. capacity of peptide spots	1×600	2×384	1.28
Max. size, mm × mm	100×150	22×60	0.09
Spot diameter, mm	2.5	0.8	0.32
Spot density, n/cm^2	4	81	20.25
Peptide density, pmol/mm^2	1,000	10	0.01
Synthesis time, weeks/50 membranes or slides	25	3	0.12
Required amount of serum, µl/n peptides	50/145	45/384	0.34
Sensitivity for Ara h 1	17/145	34/145	2.00[1]
Sensitivity for Gly m 5	8/372	162/372	20.25[2]
Specificity for Ara h 1	145/145	145/145	1.00
Specificity for Gly m 5	371/372	372/372	1.00

Parameters are determined according to the instrument configuration available and application done. Sensitivity is defined as <positive/total spots>. Specificity is defined as <negative/total spots>.

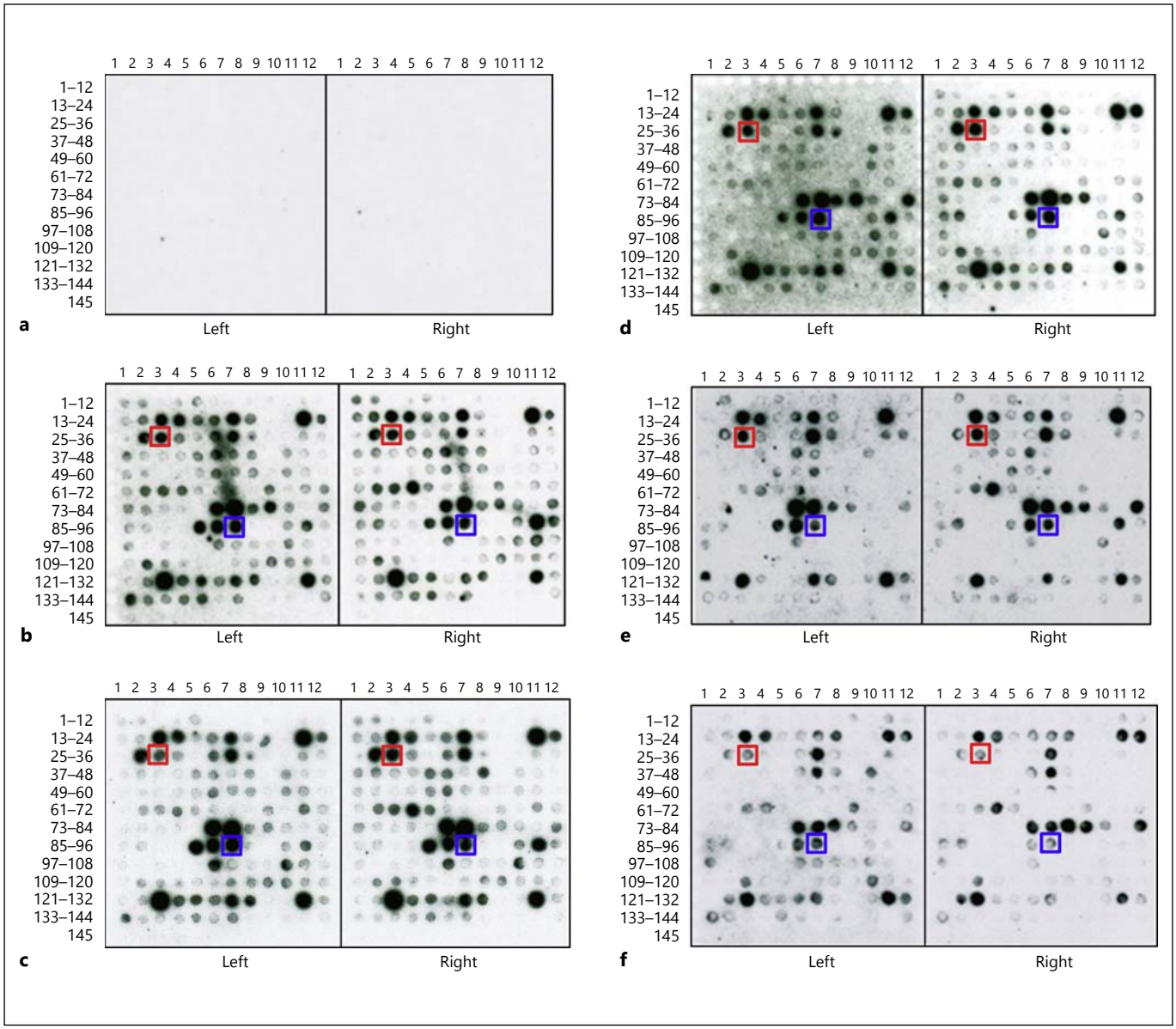
[1] 34/145 are double positive in all 5 microarray replicates; 44/145 are ≥1/2 positives in all 5 replicates; sensitivity is 2.00–2.59 times higher than that of SPOTS™.

[2] All positives of the 2 sera were counted.

segments of 145 and 372 peptide spots each, respectively. On Gly m 5 microarrays, peptides related to the subunits Gly m 5.03, Gly m 5.01 and Gly m 5.02 were spotted on positions 3–104, 106–238 and 241–377. Additional 5 spots of solid-phase cellulose without peptide were dissolved in DMSO and spotted as a control for background measurement. The negative spots were located at the spot positions 146–150 and 378–382 for Ara h 1 and Gly m 5 slides, respectively (fig. 2, fig. 5, right). Each 0.04 µl/spot were spotted according to the manufacturer's instructions (Intavis AG) at a 1.2-mm spot-to-spot distance. The spotted microscope slides were dried at 75°C for 1 h and subsequently stored under dry conditions at 4°C until use. Physical details about the peptide microarray are given in table 3.

IgE Epitope Mapping on SPOT™ Membranes

Prior to blocking, the entire cellulose membrane (150 × 100 mm) was cut into separate segments (50 × 50 mm) with each segment representing 1 protein, i.e. Ara h 1 or 1 subunit each of Gly m 5 (fig. 1, fig. 5, left). The cellulose membrane segments were blocked over night with Spots Blocking Buffer 10X (Sigma-Genosys, The Woodlands, Tex., USA) diluted 1:10 in Tris-buffered saline (TBS; 50 mM Tris, pH 7.4) containing 0.05% Tween 20 and 5% sucrose. Thereafter, each protein segment was incubated over night with 50 µl human serum diluted in 4 ml blocking buffer. For a sIgE detection, the membrane was successively incubated for 1 h each with 4 ml anti-human IgE from goat (KPL,



Color version available online

Fig. 2. Peptide microarray for the IgE epitope mapping of peanut allergen Ara h 1. IgE immunodetection of 145 overlapping synthetic peptides using a serum pool of 6 NA subjects (**a**) and serum of peanut- and soybean-allergic subject No. 28 on 5 replicate (B1–B5) slides (**b–f**) from 1 peptide synthesis batch. Each slide presents duplicate peptide segments (left/right). Consecutive numbers indicate the position of individual peptides. The peptides No. 27 and 91 are marked in red and blue colour, respectively (colours refer to the online version only).

Gaithersburg, Md., USA, via Medac, Hamburg, Germany) diluted 1:5,000 in blocking buffer and with 4 ml horseradish peroxidase-conjugated anti-goat IgG from rabbit (Dianova, Hamburg Germany) diluted 1:50,000 in blocking buffer. Finally, the membrane segments were incubated for 1 min with LumiGlo Reserve™ chemiluminescence substrate (KPL), and chemiluminescence was recorded on X-ray film by a maximum of 60-min exposure (Amersham Hyperfilm™ ECL, GE Healthcare, Little Chalfont, UK). Between all incubation steps, membrane segments were washed 4 times with TBS and 0.05% Tween 20. Positive spots of membranes incubated with human serum from allergic donors were identified visually in comparison to the control of NA serum (fig. 1, fig. 5, left).

IgE Epitope Mapping on Peptide Microarrays

Microscope slides with bound peptides were blocked for 2 h and washed as described above. Washed slides were air dried for 1 h. To the microscope slides with Gly m 5 peptides, 45 µl of undiluted hu-

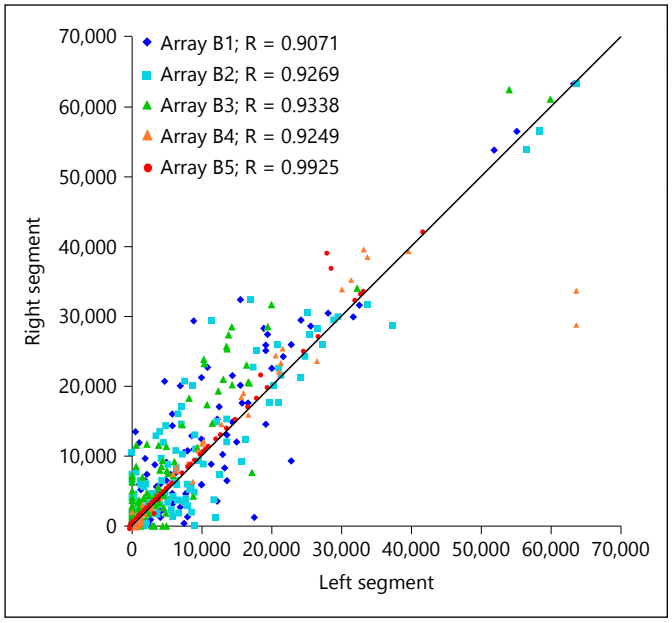


Fig. 3. Distribution of the signal intensities of IgE binding to 145 overlapping synthetic peptides of peanut allergen Ara h 1 in duplicate peptide segments (left/right) of 5 replicate (B1–B5) microarrays according to figure 2. R = Correlation coefficient.

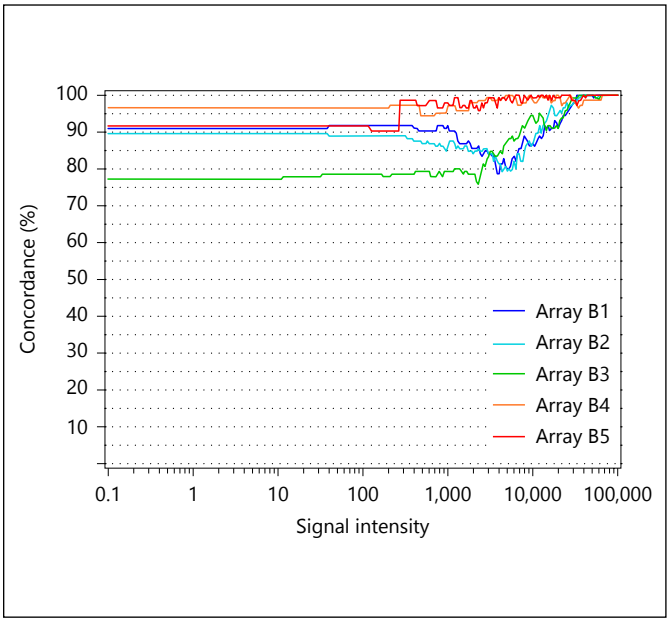


Fig. 4. Concordance, defined as the agreement between both array segments (left/right) of 5 replicate (B1–B5) Ara h 1 microarray according to figure 2 in relation to signal intensity.

man serum were applied to a glass cover slip with spacer (22 × 60 mm) (MicroArray Lifter Slips, Nunc, Germany) on top of the spotted peptide area. The serum, which was injected at the right segment and underneath the cover slip, was drawn from right to left by capillary action. To the microscope slides with Ara h 1 peptides, 29 µl undiluted human serum were applied to a smaller cover slip (22 × 40 mm) (MicroArray Lifter Slips, Nunc, Germany) as described above. The microscope slides were incubated in a wet chamber at room temperature overnight. After removing the cover slips, the microscope slides were incubated for 1 h with 4 ml anti-human IgE from goat (KPL) diluted 1:5,000 in blocking buffer following 1 h of incubation with 4 ml horseradish peroxidase-conjugated anti-goat IgG from rabbit (Dianova) diluted 1:50,000 in blocking buffer. Between all incubation steps, the membrane segments were washed 4 times with TBS and 0.05% Tween 20. After 1 min of incubation with LumiGlo Reserve™ chemiluminescence substrate (KPL), the chemiluminescence was recorded on X-ray-film within 15 min as described above. Developed X-ray films were scanned at 600 dpi on an office scanner (Hewlett Packard Scanjet 4890) and stored as 16-bit grey scale tiff. Grey scales were inverted using ImageJ software (http://imagej.nih.gov/ij/), and spots were analysed using the TIGR Spotfinder 3.1.1. software (www.tm4.com) [28]. The signal intensity of spots was calculated by the integration of unsaturated pixels in a range between 1 and 65,536 for positive spots. Using the TIGR Spotfinder 3.1.1. software, integrated spot intensity was subtracted from the background intensity which surrounded the spot (setting: circle, diameter 25, top background cut-off 15%). Spot signals were considered as positive if the background-subtracted spot intensity exceeded 4 times the average intensity of 5 negative spots, which consisted of cellulose (without bound peptide).

Statistical Evaluation of Replicate Analysis of Peptide Microarrays

Linear regression analysis of correlation plots (fig. 3) including calculation of the correlation coefficient between right and left array segments was done with Microsoft Excel 2010 for Windows.

Concordance per run was defined as agreement between both array segments, i.e. both indicating positive or both indicating negative, when moving the cut-off for positivity from low to high signal intensities. The graphical representation of concordance (fig. 4) is the percentage of concordant estimated peptides divided by all peptides assessed (n = 145) and plotted against signal intensity. The statistical analysis was performed with SAS®/STAT software, version 9.3, SAS System for Windows.

Results

Characteristics of SPOT™ Membranes versus In-House Peptide Microarrays

Table 3 summarizes various characteristics of synthesized peptides on SPOT™ membranes and microarrays. Miniaturization of the SPOT™ membrane method using the Celluspot™ array technique resulted in up to 384 peptides in 2 identical segments each on a coated microscope slide surface (22 × 60 mm) in comparison to 600 spots on the SPOT™ membranes (150 × 100 mm). While the capacity of presented peptides was comparable between

SPOT™ membranes and microarrays, the array slides measured only about one tenth of the size of a comparable SPOT™ membrane. With 81 spots/cm^2, the spot density of microarrays is approximately 20 times higher than that of the SPOT™ membranes in our synthesis configuration. A complete set of synthetic overlapping peptides (n = 372; 15-mer, 4 amino acid offsets) of the 3 subunits of the soybean major storage protein β-conglycinin, which is composed of 1,517 amino acids, was achieved on a single microscope slide (table 2). The peptide synthesis on cellulose membranes with the SPOT™ technique allows only 2 membranes to be synthesized in parallel within 1 week. This time-consuming procedure is also necessary for the microarray technique. However, after synthesis of the peptides on a modified cellulose membrane, 50 identical microarrays can be generated from 1 batch of synthesized peptides within 1 week. Taken together, peptide synthesis and spotting of 50 microarrays take 3 weeks in comparison to calculated 25 weeks to produce 50 SPOT™ membranes of 25 individual synthesis batches (table 3). In principle, this microarray technique would allow generating >1,000 slides made from 1 batch of a synthesized membrane.

For an IgE immunodetection of the soybean β-conglycinin subunits on separate cut membranes, 4 ml incubation solution each was required. Human sera were diluted 1:80 in incubation solution. Accordingly, for the detection of all 3 subunits, 150 µl undiluted human sera were required. The dilution of human serum for immunodetection of SPOT™ membranes was necessary because availability of human serum specimen in molecular allergology research is usually very much limited. By contrast, the peptide microarray covering all 3 soybean proteins required only 45 µl undiluted human serum. Thus, using the peptide microarray for IgE epitope mapping, 70% of the serum required for the detection on the SPOT™ membranes was saved. Normalized for the number of detected peptides, serum savings of 66% were achieved by the microarray (table 3). Moreover, if the SPOT™ membranes would have also been incubated with undiluted human serum according to our detection protocol, the use of our microarrays would have been 99% more efficient with regard to the required amount of serum.

Specificity and Sensitivity of IgE Binding to Synthetic Peptides on SPOT™ Cellulose Membrane versus Cellulose-Based Microarray Spots

Using peptide arrays for the purpose of linear epitope analysis of allergenic proteins, the specificity of the IgE binding should be verified. Moreover, the higher the sensitivity of the IgE binding to the synthetic peptides, the more comprehensive a screening for putative linear IgE-binding epitopes can be achieved. For a comparative analysis of specificity and sensitivity of IgE immunodetection on SPOT™ membrane versus microarray, we selected homologous high-molecular weight seed storage proteins of peanut and soybean that are considered important food allergens [24, 25]. For the verification of specificity, a NA serum pool consisting of equal volumes of sera from 6 NA human donors was applied. For investigation of the comparative sensitivity between the 2 methods, sera from donors with allergy to soybean and peanut were applied (table 1). Sera of donors with allergy to soybean and peanut as well as the NA serum pool were prescreened for the presence or absence of IgE specific to soybean, peanut, Ara h 1 and Gly m 5. All 3 peanut and soybean allergic individuals had sIgE to soybean and peanut. In addition the 3 sera had sIgE against Gly m 5, as was previously determined using an IgE-binding ELISA with nGly m 5 coated on the solid phase [25] (and unpublished data). Using ImmunoCAP analysis, elevated levels of sIgE to nGly m 5 were additionally confirmed for the sera No. 28 and 25 in this study. Serum No. 8 is assumed to have a level of sIgE against Gly m 5 comparable to that of serum No. 28 because of a similar potency of IgE binding in direct ELISA [25]. In addition, serum No. 28 had high levels of sIgE to Ara h 1. No IgE binding to peanut or soybean proteins was found in the serum pool of 6 NA donors, using Western blot or ELISA analysis (data not shown).

The specificity was calculated as the ratio between true-negative spots and the total number of spots analysed. Both on SPOT™ cellulose membranes (fig. 1a) and microarrays (fig. 2a), no false-positive detection was observed with any of the 145 Ara h 1-related peptides using the NA serum pool. Similarly, the NA serum pool showed no binding to any of the Gly m 5 peptides, except for 1 false-positive binding to peptide 35 of Gly m 5.03, on SPOT™ membranes (fig. 5a, left). No false-positive detection was observed with any of the Gly m 5 subunits on the microarray (fig. 5a, right). In summary, IgE detection of both methods of peptide presentation showed a comparable high specificity of 100% (table 3).

The sensitivity was calculated as the ratio of detected spots and the total number of spots analysed. Using serum No. 28, 17 peptides of Ara h 1 bound IgE on a SPOT™ membrane (fig. 1b). In comparison, 32 peptides of Ara h 1 located on the microarray bound IgE from serum No. 28 in both segments of all 5 replicate runs (fig. 2b–f). Of the

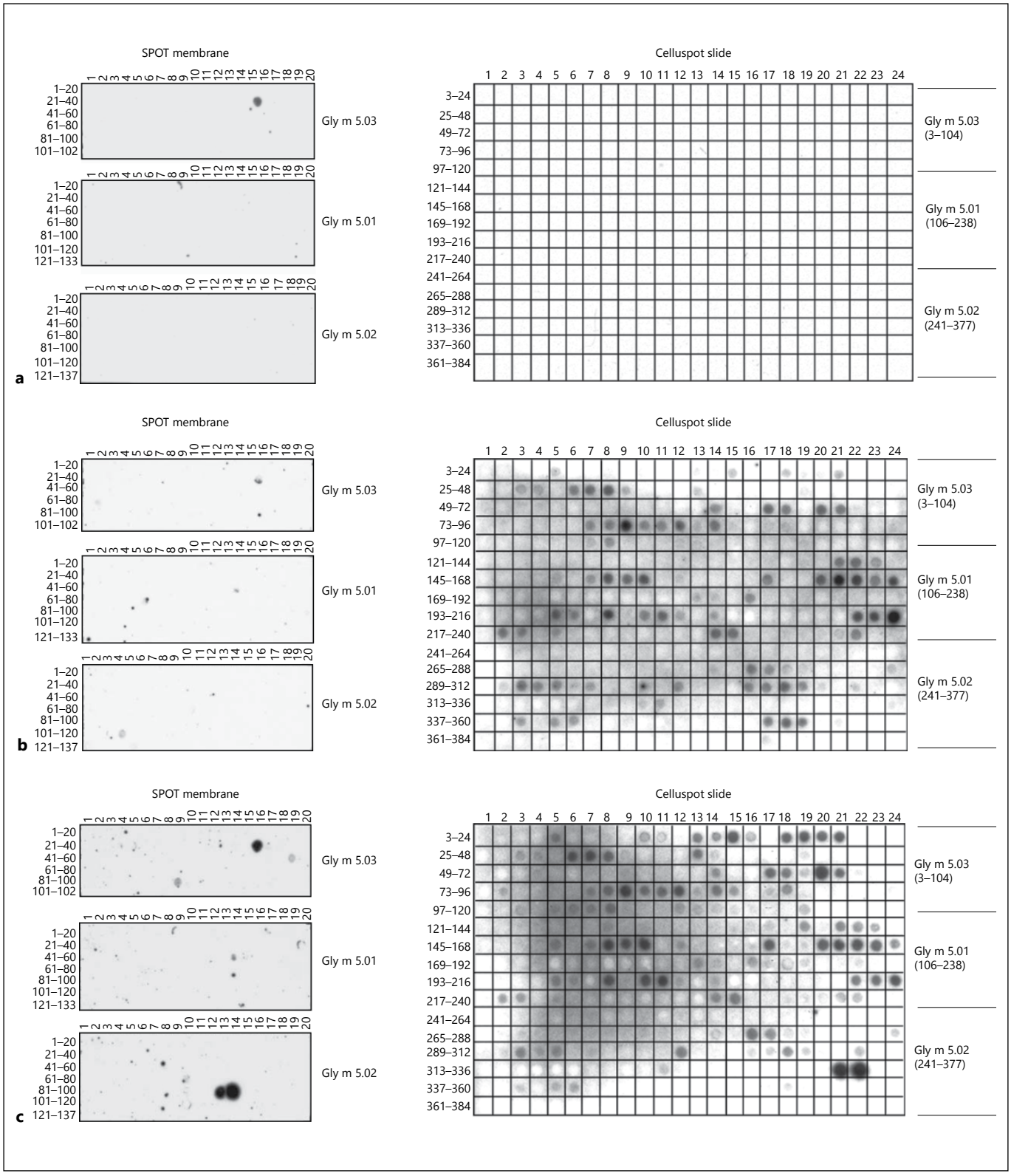


Fig. 5. Comparison of IgE binding to 372 overlapping synthetic peptides of the 3 soybean β-conglycinin subunits Gly m 5.03, 5.01 and 5.02 on SPOT™ membranes (left) versus microarrays (right; display of one segment only). **a** Serum pool of 6 NA subjects. **b** Serum of soybean allergic subject No. 8. **c** Serum of soybean allergic subject No. 25.

17 peptides identified on the SPOT™ membrane, 15 were detected in both segments of all 5 replicate arrays, and another 2 were detected in both segments of each 4/5 array. In summary, all IgE-binding peptides on the SPOT™ membrane were detected in >97% (83/85) of identical peptide duplicates on microarrays. Moreover, 44 peptides were considered as IgE binding on microarrays if ≥1/2 positives in all 5 replicates was taken as a criterion for positivity. Consequently, the detection of IgE-binding peptides of Ara h 1 with serum No. 28 on microarrays was approximately twice as sensitive as on a SPOT™ membrane. Moreover, the sensitivity of IgE binding to synthetic overlapping peptides was investigated for the 3 subunits of Gly m 5 using the sera No. 8 and 25 on SPOT™ membranes (fig. 5b, c, left) and microarrays (fig. 5b, c, right). On peptide microarrays, only one of the 2 segments was analysed because of the overall good concordance of detection between the right and the left segment as described below. In SPOT™ analysis, IgE binding to peptide 35 of Gly m 5.03 is considered non-specific because of a positive detection in both sera of soy-allergic donors as well as in the NA control. By contrast, this peptide did not bind IgE in microarray analysis. The peptides 93 and 94 of Gly m 5.02 showed intense IgE binding in both SPOT™ and microarray analysis. Additional weak IgE binding in SPOT™ analysis was not confirmed in microarray analysis. We assume that the reactivity was caused by an unspecific immunodetection on the SPOT™ membrane because unspecific IgE binding was only seen in the NA controls of the immunodetection of the SPOT™ membranes but not of the microarrays. Including those presumably false-positive peptides on SPOT™ membranes, 8/372 peptides bound IgE from both sera of soy-allergic donors on SPOT™ membranes in comparison to 162/372 peptides on microarrays (table 3). Thus, the detection of IgE binding to Ara h 1- and Gly m 5-related peptides was approximately 2–20 times more sensitive on our microarrays. A comparable increase in sensitivity was observed for the IgE detection of Gly m 5-related peptides on our microarrays using serum No. 28, and for the IgE detection of Ara h 1-related peptides using the sera No. 8 and 25 (data not shown).

Reproducibility and Concordance of IgE Binding to Peptides on Microarrays

The microarray analysis of IgE binding to synthetic overlapping peptides of peanut Ara h 1 was performed on 5 replicate slides using serum No. 28 (fig. 2b–f). Measured intensities of IgE binding of the 5 replicates are displayed in figure 3 as a correlation between the right and left segments. Linear regression of signal intensities of IgE binding in right versus left segments resulted in a mean slope of 0.9958 (range 0.8007–1.1596) and a mean correlation coefficient of 0.9370 (range 0.9071–0.9925). With an average intercept of +928 (range 73–1,462) intensity counts of the right segment (y-axis), the IgE detection was slightly more sensitive in the right segment in which the serum was applied. Taken together, the signal intensities between the right and left segments were very much comparable.

Further, the intra-array concordance of both positive or both negative results of IgE binding between the 2 segments was calculated for the 5 replicates. The concordance depending on signal intensity is displayed in figure 4. The average concordance at a signal intensity ≥1 was >80% (runs B1 = 91%, B2 = 90%, B3 = 77%, B4 = 97%, B5 = 92%).

Increasing the signal intensity cut-off for positive signals above 10,000 resulted in >85% concordance for both positive or both negative results of IgE binding in all 5 runs. As examples, positive signals above 10,000 are found for the peptides No. 27 (12,009–25,083 signal intensity) or 91 (10,485–25,943 signal intensity) in both segments of all 5 replicate slides (fig. 2b–f). It should be noted that at higher signal intensities for a cut-off between negative and positive results, the most concordant results are concordant negative results. In addition, and as described above, 17 peptides which bound IgE in SPOT™ analysis bound IgE in >98% (167/170) of identical peptide replicates on our microarrays and in >97% of positive duplicate segments.

Discussion

The mapping of linear IgE-binding epitopes on the basis of synthetic overlapping peptides, which cover the complete primary protein structure, is a common tool to investigate B-cell epitopes of allergens. The SPOT™ membrane technique, which was introduced by Frank and Overwin [11], was applied for human IgE epitope mapping of various food allergens [5–7, 12, 29, 30], including peanut Ara h 1 [29] and soybean allergens other than Gly m 5 [30, 31]. However, this technique is time consuming and requires high amounts of limited available human sera. Because of these obvious limitations, peptide microarrays have recently been developed and used for allergen epitope identification, like in other fields of nucleic acid [32], protein/allergen [33] and peptide [34] research. Selected examples of published microar-

rays with synthetic overlapping peptides for human IgE epitope mapping of food allergens were on milk lactoglobulin and casein subunits [20, 35], egg ovomucoid [36], peanut and walnut allergens [37, 38], including Ara h 1 [37], shrimp allergens [39] and Atlantic salmon and cod parvalbumins [40, 41]. Most of the published and above-cited microarray-based analysis of linear IgE epitopes of food allergens is based on 1 previous microarray development for milk allergens [20]. In comparison to other published work, the IgE microarray development by Lin et al. [20] has been the most comprehensively validated in the field of food allergens to our knowledge. However, comparability of performance and results to the previous SPOT™ membrane technique was limited to published data which were obtained by using different patients' sera. In this work, we present the development of a novel multipeptide microarray and its validation in direct experimental comparison to the SPOT™ membrane technique for linear IgE epitope mapping. The novel microarray is based on the Celluspot™ array technique that makes use of peptides covalently synthesized on a cellulose porous membrane. Upon dissolution in DMSO, the cellulose with covalently bound peptide builds a 3-dimensional structure that is spotted and attached onto an adhesive foil surface of a standard microscope slide. The slides generated by this procedure were stable for 1 year at 4°C under dry storage conditions (data not shown). Each slide had a capacity to present 384 peptides in duplicate segments, which was appropriate to present overlapping peptides (15-mer, 4 amino acid offsets) of the high-molecular weight soybean allergen Gly m 5 that is composed of three 50–71 kDa subunits [42]. We investigated the IgE binding of 3 individual sera, donated from soybean- and peanut-allergic individuals, to 4 individual vicilin-like proteins, namely peanut Ara h 1 and the 3 soybean Gly m 5 subunits. In our study, we observed a very high specificity of IgE detection to synthetic peptides using both SPOT™ membranes and microarray slides. We achieved an increase in sensitivity between 2- and 20-fold using the microarray, while the required amount of human sera was reduced by at least two thirds over the SPOT™ membrane technique (table 3). With a total volume of 50 µl of serum to study in detail, the IgE binding to a high-molecular weight allergen such as Gly m 5 makes the cellulose-based peptide microarray an ideal tool for studying populations on the individual subject level with limited availability of serum specimen, such as paediatric study groups. Having in mind the focus of a primary screening for potential linear IgE epitopes of allergens that would require further verification on the level of a full-length protein, our novel peptide microarray presented appropriate and acceptable reproducibility above an average concordance of >80% between duplicate intra-array detection and for multiple interarray detection of the most prominent IgE-binding peptides. Based on the theoretical synthesis of 50 identical peptide batches using the SPOT™ membrane technique, the generation of 50 identical microarrays from 1 peptide synthesis batch was calculated to be 8 times faster (table 3). Further, considering the possibility to potentially generate >1,000 slides from 1 peptide synthesis batch, this microarray technique is appropriate to investigate large study groups on the individual level. In summary, we found the cellulose-based microarray superior to the SPOT™ membrane technique in many ways. The performance of the peptide microarrays appeared to be similar to that of the previously published peptide microarray for milk allergens [20]. While we applied 50 µl of undiluted serum to 1 array, Lin et al. [20] used 250 µl of 1:5 diluted serum. The purity of synthesized peptides was estimated between 70 and 80% for both microarrays, and IgE bound to peptides was detected using a polyclonal antibody directed against human IgE. Lin et al. [20] reported a specificity of >96% with regard to less than 10/289 peptides that showed non-specific binding. Using our microarray, we did not observe non-specific binding to any of the 145 and 372 presented peptides, respectively. Differences between both microarray systems are identified in the synthesis of peptides, preparation of peptide microarrays and recording of signals of IgE binding to peptides. We were able to perform in-house multiple peptide synthesis in comparison to the use of commercially synthesized peptides by Lin et al. [20]. In our hands, this turned out to be a very flexible and cost-effective option. Our in-house synthesized peptides were covalently linked to a 3-dimensional cellulose polymer structure which allows a high-density presentation of peptides in comparison to monolayer printing on modified glass slides (Intavis personal communication). Considering the low concentration of sIgE at the ng/ml level in comparison to potentially competitive sIgG at the µg/ml level, a polymeric high-density presentation of peptides, at least in theory, may be advantageous over a monolayer peptide presentation. The published microarray further required an array reader for signal recording, while our method of detection required low-tech chemiluminescence exposure on X-ray film and digitalization on a standard desktop scanner. Independently, the recorded microarray signals were accessible for open access array software for spot identification and analysis.

Conclusion

With special regard to using our method as a research tool, we have developed and established a novel, simple, cost-effective, sensitive and specific peptide microarray for the investigation of potential IgE epitopes of peanut and soybean allergens in large study groups, including paediatric individuals of whom the amount of available serum donations is usually very much limited. Likewise, the method may be applied to discover peptides that bind IgG from human serum. The identification of sIgE and IgG-binding patterns can contribute to the development of novel diagnostic and therapeutic approaches in food allergy and provides basic information on epitopes with regard to the allergen risk assessment of novel proteins or foods derived from novel natural food sources or biotechnology.

Acknowledgements

We thank Dr. Ole Brand (Intavis AG) for his excellent technical support.

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